

A comparison between the effectiveness of the ethanol based extract of pomegranate peel (*Punica granatum*) and simvastatin drug for lowering blood LDL level in hypercholesterolemic male rats (*Rattus novergicus*)

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ABSTRACT

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Background: Hypercholesterolemia is one of the major risk factors for coronary heart disease. Drugs of natural pomegranate peel extract using ethanol can be used as an alternative of simvastatin as standard drugs. Preclinical trials are needed before clinical trials in humans to ensure the efficacy of the drug from the nature

Objective: To compare effectiveness of pomegranate peel extract using ethanol (*Punica granatum*) with simvastatin for lowering LDL level in hypercholesterolemia white rats (*Rattus novergicus*).

Methods: This study used an experimental research design with pretest-posttest control group. We use 27 male hypercholesterolemia wistar strain rats were used as samples. The samples were divided into 3 groups. Group I as a negative control was given DMSO 2% 2ml/300gramBW, group II was given simvastatine 0.18 mg/200gramBB/day, group III was given ethanol extract of pomegranate peel 30mg/200gramBB/day for 15 days

Results: Results of paired t-test showed that the group treated with simvastatin decreased LDL levels significantly. The group treated with ethanol extract of pomegranate peel decreased LDL levels significantly. The group given the ethanol extract did not decrease LDL levels significantly.

Conclusion: Extract of pomegranate peel using ethanol is not effective for lowering LDL levels in hypercholesterolemia white rats (*Rattus novergicus*) compared to simvastatin.

Latar Belakang: Hiperkolesterolemia merupakan faktor risiko mayor penyakit jantung koroner. Obat dari alam ekstrak etanol kulit delima dapat dimanfaatkan sebagai alternatif terapi simvastatin sebagai obat standard. Uji preklinik diperlukan sebelum uji klinik pada manusia untuk memastikan khasiat obat dari alam tersebut.

Tujuan Penelitian: Untuk mengetahui perbandingan efektifitas antara pemberian ekstrak etanol kulit delima (*Punica granatum*) dengan simvastatin terhadap penurunan kadar LDL darah pada tikus putih (*Rattus norvergicus*) hiperkolesterolemia.

Metode Penelitian: Penelitian eksperimental ini menggunakan pre-test dan post test control group design pada 27 tikus galur Wistar jantan hiperkolesterolemia. Subjek dibagi menjadi 3 kelompok. Kelompok I sebagai kontrol negatif (DMSO 2% dosis 2ml/300gramBB), kelompok II diberikan simvastatin dosis 0,18 mg/200gramBB/hari, kelompok III diberi ekstrak etanol kulit delima dosis 30mg/200gramBB/hari selama 15 hari.

Hasil: Hasil uji t-test berpasangan menunjukkan bahwa kelompok yang diberi perlakuan simvastatin mengalami penurunan kadar LDL secara signifikan. Kelompok yang diberi ekstrak etanol tidak mengalami penurunan kadar LDL yang signifikan.

Kesimpulan: Pemberian ekstrak etanol kulit delima (*Punica granatum*) tidak efektif menurunkan kadar LDL darah tikus putih (*Rattus norvegicus*) hiperkolesterolemia dibandingkan dengan simvastatin.

INTRODUCTION

Hypercholesterolemia is an increase of fasting LDL cholesterol without elevated triglyceride levels. American Heart Association (AHA) estimates that more than 100 million Americans have total cholesterol levels ≥ 200 mg/dl, which were categorized under high enough. The prevalence of hypercholesterolemia in Indonesia at the age group 25-34 years was 9.3%. This amount increased by 15.5% at the age group 55-64 years. Hypercholesterolemia is generally more common in women (14.5%) than men (8.6%). Hypercholesterolemia is one of the major risk factors for coronary heart disease (CHD). World Health Organization (WHO) estimates that hypercholesterolemia is associated with more than half the incidence of coronary heart disease and more than four million deaths every year.¹

Indonesian Society of Endocrinology (PERKENI) formulates that the treatment of hypercholesterolemia are non-pharmacologic therapy such as lifestyle changes and pharmacological or use of cholesterol-lowering drugs. First line drug which is recommended by the NCEP-ATP III (National Cholesterol Education Program- Adult Treatment Panel III) is the class of HMG-CoA reductase inhibitor. One of the examples of stain group is simvastatin, which is currently the most effective hypolipidemic for lowering cholesterol.¹

In addition to using chemical drugs, drugs

of natural origin can be used to lower the blood LDL level. One of the natural medicinal drug is pomegranate juice. Administration of pomegranate juice can lower the LDL levels in patients with type 2 diabetes mellitus accompanied with hypercholesterolemia.² Other studies also showed that the ethanol extract of pomegranate skin in hypercholesterolemia rats lowers the blood LDL level.³

Traditional medicine in order to be accepted to formal health care/medical profession, the results of empirical data must be backed up by scientific evidence of the efficacy and safety of its use for humans. Experiences supported by empirical research is increasingly given confidence on efficacy and safety of traditional medicines.⁴ Such evidence can be obtained from systemically conducted studies. Stages of development of traditional medicine into phytopharmacy are selection, preclinical testing (toxicity and pharmacodynamic test), standardization and clinical trials.⁵

METHODS

The study was conducted using an experimental pre-test post-test with control group (pre-test post-test control group design). The study was conducted at the Laboratory of Integrated Research Center (LPPT) Gadjah Mada University, Yogyakarta in January-March 2014. This study was approved by the Committee of Ethical Research at the Medical Faculty of Medicine and Health, University of Muhammadiyah Yogyakarta.

The study population was 3 months aged male Wistar rats around 200-250 gram of weight.³ Rats were divided into 3 groups after induction. The number of subject was taken by formula Federer. The total number of subject was 27 animals, where each of the group consisted of 9 rats.

Table 1 Distribution of each group and treatment

Group	Treatment
1	DMSO 2% solvent were given and considered as control
2	0.18 mg/200gramBW of Simvastatin per day were given orally
3	Ethanol extract of pomegranate peel 30mg/200gramBW were given per day orally

Induction

Before the induction of diabetes in rats, they were adapted for one week at room temperature with adequate humidity and lighting condition. Furthermore, the weight of each rat was measured to determine the induction dose. Glucose monohydrate at a dose of 1,125 mg/200gram BW orally once a day was used as provision of induction dose throughout the 45 days.³ High-fat feed AD2 and lard were given to rats with a ratio of 9:1 provided in ad libitum. The Given lard was stopped after an induction period. During 45 days Total cholesterol level of rat was checked after induction (pre-test). Rat s with total cholesterol level more than 54 mg/dL was devined as hypercholesterolemia.⁶ The rats were divided into three groups randomly.

Treatment

The classified three groups were given different treatment according to respective group.³ The treatment was carried out throughout 15 days.

Examination of blood LDL levels

Having anaesthetized with ketamine, blood sampling of samples was executed using a capillary tube at the retro orbital plexus. Blood was collected in 0.5 ml eppendorf tubes. Total cholesterol was determined by CHOD-PAP method. Blank was made with 1000 microliter CHOD-PAP reagent. Standard solution was made with 1000 microliters of reagents and 10 microliters standard. The sample solution was made using 1000 microliter reagent and 10 microliters of samples (plasma that had been

centrifuged). Each solution was then incubated for 5 minutes at a temperature of 250C. After incubation the absorbance value was read at a wavelength of 546nm. LDL of cholesterol was determined using precipitation or deposition method. LDL cholesterol was calculated from the difference in the supernatant with total cholesterol. One hundred micro-sample was mixed with 1000 microliter LDL reagents. Samples were allowed to stand for 10 minutes at a temperature of 15-25°C and then centrifuged for 10 min at 4000 rpm. The formed supernatant was taken to make the reference solution, which is a mixture of 100 microliters of supernatant and 1000 microliters of CHOD-PAP reagent. Samples were incubated again for 5 minutes at a temperature of 25°C and absorbance was read at a wavelength of 546 nm.⁶

Data Analysis

All results were explained with mean value and standard deviation. The normal distribution of data was tested by the Shapiro-Wilk. Test differences between groups were tested by One Way ANOVA for analysis of total cholesterol and Kruskal Wallis test for analysis of LDL levels. Analysis of differences in the data pre-test and post-test was analyzed using paired T-test.

RESULTS

The total cholesterol levels pretest result was checked the next day after having induced rats. Blood samples were taken from orbital sinus vein of rat. Having obtained the samples of blood serum total cholesterol was then checked by spectrophotometric method. Data of pre-test total cholesterol level showed that rats were experienced hypercholesterolemia (Table 2). Being confirmed that rats experienced hypercholesterolemia, then the treatment was applied on rat in accordance with pre-defined group. Treatment was given for 15 days. Having given treatment on all groups, total cholesterol levels of rat was examined as post-test data (Table 2).

Blood LDL levels of pre-test and post-test were obtained after examination of total cholesterol level. Level of LDL is shown in Table 3.

Table 2 The mean total cholesterol levels of pre-test and post-test

Treatment group	Total cholesterol level (mg/dL) \pm SD		p*	The average decrease percentage of total cholesterol levels
	(Pre-Test)	(Post-Test)		
Control	190,9 \pm 4,0	190,3 \pm 4,1	0,042	0,10 \pm 0,37
Simvastatin	59,1 \pm 5,5	53,6 \pm 7,9	0,210	9,07 \pm 13,08
Extract of Pomegranate peel using ethanol	62,9 \pm 5,2	64,1 \pm 10,4	0,664	-1,04 \pm 8,21
p**				0,188

values are mean \pm SD

* Paired t-test significant if $p < 0,05$

** One Way ANOVA test significant if $p < 0,05$

Table 3 The mean blood LDL levels of pre-test and post-test

Treatment group	LDL level (mg/dL) \pm SD		p*	The average decrease percentage of blood LDL levels
	(Pre-Test)	(Post-Test)		
Control	70,2 \pm 1,7	70,2 \pm 2,0	0,941	-0,05 \pm 0,78
Simvastatin	20,7 \pm 2,8	13,4 \pm 1,9	0,008	34,19 \pm 13,41
Extract of Pomegranate peel using ethanol	21,4 \pm 2,2	22,6 \pm 9,2	0,736	0,90 \pm 23,26
p**				0,023

values are mean \pm SD

* Paired t-test significant if $p < 0,05$

** One Way ANOVA test significant if $p < 0,05$

DISCUSSION

Total cholesterol levels

It can be seen from the Table 2 that there is a decrease of total cholesterol levels in the control group and simvastatin. While, the group of pomegranate peel using ethanol extract total cholesterol levels was increased.

One way Anova parametric test showed that there is no significant difference in the group 1 with other groups for lowering total cholesterol levels.

Differences of data pre-test and post-test each group were tested with paired t-test (paired-sample t-test). Based on the unpaired t-test in each treatment group, showed that the treatment

group given 2% DMSO control demonstrated a significant reduction. Simvastatin group showed no significant reduction. While the ethanol extract of pomegranate skin showed an insignificant increase.

The given DMSO inhibits the atherosclerosis induction by cholesterol.⁷ In this study it was expected that the ethanol extract of pomegranate skin would decrease to an average level of total cholesterol as research conducted by Althunibat (2010) administration of the extract of pomegranate peel using methanol can lower the total cholesterol levels.⁸ Polyphenols contained in the pomegranate peel extract can lower serum lipids.⁹ It was expected that the results of this research would be influenced by various factors and drug response. The effect

of therapy or drug response of an individual is influenced by various factors such as sex, age, body weight, genetic factors, psychological factors, drug resistance, the environment and disease.¹⁰ In this study, total cholesterol were not examined before the induction of glucose monohydrate and high-fat diets so it was unsure whether the rats previously had been induced by hypercholesterolemia. The amount of diet per day rat was not counted. Therefore, the required amount of daily diet for rats was not determined. Differences in diet of rat affect the measured cholesterol level. Other factors that can affect is the differences of metabolism in an individual animal, the differences of response in the experimental animals at the time of dietary cholesterol and the amount of food consumed by the tested animals.¹⁰ The amount of rat diet may affect the results of this study. The test animals testing were likely to experience emotional distress (stress) during the treatment so that it can affect the obtained results.¹⁰

LDL Level of Blood

Data from post-test to measure blood LDL levels indicate that there is a decrease of blood LDL levels in the simvastatin group. Rats given extract of pomegranate peel using ethanol showed an average increase in blood LDL levels. There is no different on average level of cholesterol LDL between pretest and post-test result in group 1. The data regarding the decreased levels of blood LDL is shown in Table 2 in the form of percentages. The non-parametric test of Kruskal-Wallis showed that there were significant differences the effect of lowering blood LDL levels of at group I to another group. The results of Mann Whitney Test showed that simvastatin group showed significant differences compared to other groups, whereas the control group showed no significant difference with the group of extract of pomegranate peel using ethanol.

Differences of data pre-test and post-test each group were tested with paired t-test (paired-sampled t-test). Based on the unpaired t-test in each treatment group, showed that all treatment groups were given 2% DMSO control

demonstrated a significant reduction. Simvastatin group showed no significant reduction. While the ethanol extract group showed insignificant improvement.

The extract of pomegranate peel using ethanol showed better performance for reducing blood LDL levels than DMSO 2% due to the effect of the active chemical compounds derived from ethanol based extract of pomegranate peel.

Due to extraction using ethanol several chemical compounds containing in pomegranate rind becomes active as for example tannins and flavonoids.¹³ Such compounds have hypolipidemic effect by different mechanisms. The results of this research has similarity with the research conducted by Fatma (2009) in which extract of pomegranate using ethanol solvent showed lower blood LDL levels compared to control group. In this study, initially it was estimated that the effects extract of pomegranate peel using ethanol solvent better than simvastatin. However, the differential effect of control group in this study was not significant. The study conducted by Wei et al. (2011) reported that the effects of extract of pomegranate peel using ethanol is equivalent to the positive control.¹² Limitations of this study was not to know the exact amount of each active ingredient resulting from the extraction process, which is responsible for lowering blood LDL levels. The duration of the storage period of extract is another factor that may affect the results of this study. This study uses only a single dose administration. The study conducted by Fatma (2009) during extraction of pomegranate peel, various dosages of ethanol were used to determine the differences in the effectiveness of each dosage on reducing blood LDL levels.

In this study the given simvastatin reduced blood LDL levels and inhibited HMG CoA reductase which inhibited HMG CoA into mevalonate. Therefore the synthesis of cholesterol was inhibited, which led to a decrease in the concentration of cholesterol in the liver cells and increased the LDL receptor (E, Apo-B-100). The treatment groups using simvastatin in the study had the most excellent

effect of lowering LDL than other groups and had significant differences.²

CONCLUSIONS

Extract of pomegranate peel using ethanol is not effective for lowering LDL levels in hypercholesterolemia white rats (*Rattus norvegicus*) in comparison with simvastatin. Further research is needed on various active compounds contained in the pomegranate tree (fruits, seeds, leaves, stems, flowers or roots) which might be served to lower the blood LDL level. Besides, in-depth study on the usage of various solvents for extraction purposes should be practiced. Dose variations and toxicity test of usage of ethanol for extraction should also be considered in future.

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